Research Article

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Thymic NF-κB-inducing kinase regulates CD4+ T cell-elicited liver injury and fibrosis in mice

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Background & Aims: The liver is an immunologically-privileged organ. Breakdown of liver immune privilege has been reported in chronic liver disease; however, the role of adaptive immunity in liver injury is poorly defined. Nuclear factor-κB-inducing kinase (NIK) is known to regulate immune tissue development, but its role in maintaining liver homeostasis remains unknown. This study aimed to assess the role of NIK, particularly thymic NIK, in regulating liver adaptive immunity.

Methods: *NIK* was deleted systemically or conditionally using the Cre/loxp system. Cluster of differentiation [CD]4+ or CD8+ T cells were depleted using anti-CD4 or anti-CD8 antibody. Donor bone marrows or thymi were transferred into recipient mice. Immune cells were assessed by immunohistochemistry and flow cytometry.

Results: Global, but not liver-specific or hematopoietic lineage cell-specific, deletion of *NIK* induced fatal liver injury, inflammation, and fibrosis. Likewise, adoptive transfer of *NIK*-null, but not wild-type, thymi into immune-deficient mice induced liver inflammation, injury, and fibrosis in recipients. Liver inflammation was characterized by a massive expansion of T cells, particularly the CD4+ T cell subpopulation. Depletion of CD4+, but not CD8+, T cells fully protected against liver injury, inflammation, and fibrosis in *NIK*-null mice. NIK deficiency also resulted in inflammation in the lung, kidney, and pancreas, but to a lesser degree relative to the liver.

Conclusions: Thymic NIK suppresses development of autoreactive T cells against liver antigens, and NIK deficiency in the thymus results in CD4+ T cell-orchestrated autoimmune hepatitis and liver fibrosis. Thus, thymic NIK is essential for the maintenance of liver immune privilege and liver homeostasis.

Lay summary: We found that global or thymus-specific ablation of the *NIK* gene results in fatal autoimmune liver disease in mice. NIK-deficient mice develop liver inflammation, injury, and fibrosis. Our findings indicate that thymic NIK is essential for the maintenance of liver integrity and homeostasis.

Keywords: Liver injury; Inflammation; Liver fibrosis; CD4-positive T-lymphocytes; Hepatitis, autoimmune; Liver disease; Flow Cytometry; Liver cirrhosis.

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Introduction

The liver is an immunologically-privileged organ. However, liver self-tolerance is compromised in autoimmune liver diseases, including primary biliary cirrhosis, primary sclerosing cholangitis, and autoimmune hepatitis (AIH).[1,2] Hepatic infiltration by T cells, particularly the cluster of differentiation [CD]4+ subpopulation, is reported to be associated with alcoholic liver disease, non-alcoholic steatohepatitis (NASH), and hepatotoxin-induced chronic liver injury in rodents and humans;[3–7] however, the underlying mechanism responsible for the breakdown of liver immunological privilege is largely unknown. The contribution of adaptive immunity to liver disease progression is also poorly understood.

Nuclear factor-κB-inducing kinase (NIK), also called MAP3K14, mediates activation of the noncanonical NF-κB2 pathway in response to a subset of cytokines.[8,9] NIK phosphorylates and activates IKKα that in turn phosphorylates NF-κB2 p100 precursors.[10,11] Phosphorylation of p100 induces ubiquitination of p100, resulting in proteolytic cleavage of p100 precursors to generate transcriptionally-active NF-κB2 p52 isoform.[11] Functionally, NIK regulates lymphoid tissue development and adaptive immunity in mice.[12–14] We recently reported that NIK also regulates hepatocyte-Kupffer cell (KC) crosstalk in the liver.[15] Of notice, a homozygous loss of function *NIK* mutation in humans is associated with abnormal immunity as well as liver dysfunction,[16] raising the possibility that NIK regulation of immunity and liver function may be conserved in humans.

In this study, we characterized global as well as tissue-specific *NIK* knockout (KO) mice. We found that whole body, but not liver-specific or hematopoietic lineage cell-specific, *NIK* KO mice develop fatal liver inflammation, injury, and fibrosis. Likewise, NIK deficiency in the thymus also results in autoimmune liver disease. We further demonstrated that in *NIK* KO mice, CD4+ T cells orchestrate immune attacks against liver.

Materials and methods

Generation of NIK KO mice

Animal experiments were conducted following the protocols approved by the University of Michigan Institutional Animal Care and Use Committee. Two loxp sites were inserted into 2 *NIK* introns (NIK^{flox}). To generate whole body *NIK* KO mice ($NIK^{-/-}$), NIK^{flox} mice were crossed with *EIIA*-Cre drives, in which Cre was expressed in germlines,[17] to generate $NIK^{+/-}$ mice ($NIK^{flox/+}$; *EIIA*-Cre). $NIK^{+/-}$ mice were backcrossed with C57BL/6 wild-type (WT) mice for >6 generations to eliminate *EIIA*-Cre. To generate hepatocyte-specific or myeloid cell-specific *NIK* KO mice, $NIK^{flox/flox}$ mice were crossed with *albumin*-Cre or *lysM*-Cre drivers, respectively. Mice were housed on a 12-h light–dark cycle and fed a normal chow diet (9% fat; Lab Diet, St. Louis, MO) *ad libitum* with free access to water.

Adoptive transfer of bone marrow cells

WT or KO recipient males (5 weeks) were pretreated with $GdCl_3$ (i.p. 10 mg/kg body weight two times at a 4-day interval) and lethal irradiation (2 × 6 Gy, 3 h apart), and then received donor bone marrow cells (2 x 10^6 cells/mouse) via tail vein injection (6 h after irradiation). Donor bone marrow cells were harvested from the femurs and tibias of WT or KO mice (5 weeks) and depleted of red blood cells (RBCs) using a RBC lysis buffer (NH_4Cl 155 mM, $KHCO_3$ 10 mM, EDTA 0.1 mM, pH 7.3). Recipients drank acidic water (pH 2.6) during $GdCl_3$ treatments and for additional 2 weeks (supplemented with 0.1 mg/ml neomycin) after bone marrow transplantation.

Thymus transplantation

Donor thymi were isolated from WT or *NIK* KO male littermates (5 weeks). $Foxn1^{nu}$ male recipients (5 weeks) (Stock No: 002019, Jackson laboratory) were anesthetized with isoflurane. A midline incision was made to expose kidney on the left side, and donor thymus (25 mg) was placed under renal capsules. The incision was sutured, and health conditions were monitored daily.

Anti-CD4 or anti-CD8 antibody treatment

Mice (3 weeks) were intraperitoneally injected with anti-CD4 (GK1.5; BioXCell, BE0003-1) or anti-CD8 (YTS169.4; BioXCell, BE0117) antibody (100 μg/mouse) weekly for three consecutive weeks.

Blood analysis

Blood glucose and alanine aminotransferase (ALT) activity were measured using glucometers (Bayer Corp., Pittsburgh, PA) and an ALT reagent set (Pointe Scientific Inc., Canton, MI), respectively.

Hepatocyte and leukocyte isolation

Primary hepatocytes were prepared from mouse liver using type II collagenase (Worthington Biochem, Lakewood, NJ).[18] To isolate leukocytes, blood samples were collected from tail vein using heparin-coated capillaries and centrifuged at 2000 rpm for 10 min at room temperature. Leukocyte pellets were washed three times with RBC lysis buffer.

Real-time quantitative PCR (qPCR)

Total RNAs were extracted using TRIzol reagents (Life technologies). Relative mRNA abundance of different genes was measured using SYBR Green PCR Master Mix (Life Technologies, 4367659).

Immunoblotting

Tissue samples were homogenized in lysis buffer (50 mM Tris, pH 7.5, 1% Nonidet P-40, 150 mM NaCl, 2 mM EGTA, 1 mM Na_3VO_4, 100 mM NaF, 10 mM $Na_4P_2O_7$, 1 mM benzamidine, 10 μg/ml aprotinin, 10 μg/ml leupeptin; 1 mM phenylmethylsulfonyl fluoride). Proteins were separated by SDS-PAGE and immunoblotted with the indicated antibodies.

Hydroxyproline assays

Liver samples were homogenized in 6 N HCl, hydrolyzed at 100°C for 18 h and centrifuged at 10000 rpm for 5 min. Supernatant was dried in speed-vacuum, dissolved in H_2O, and neutralized with 10 N NaOH. Samples were incubated in a chloramine-T solution (60 mM chloramines-T (Sigma, 857319), 20 mM citrate, 50 mM acetate, pH 6.5) for 25 min at room temperature, and then in Ehrlich's solution (Sigma, 038910) at 65°C for additional 20 min. Hydroxyproline content was measured using a Beckman Coulter AD 340 Plate Reader (570 nm) and normalized to liver weight.

Reactive oxygen species assays

Liver lysates were mixed with a dichlorofluorescein diacetate fluorescent (DCF, Sigma, D6883) probe (5 μM) for 1 h at 37°C. DCF fluorescence was measured using a BioTek Synergy 2 Multi-Mode Microplate Reader (485 nm excitation and 527 nm emission).

Immunostaining

Liver frozen sections were prepared using a Leica cryostat (Leica Biosystems Nussloch GmbH, Nussloch, Germany), fixed in 4% paraformaldehyde for 30 min, blocked for 3 h with 5% normal goat serum (Life Technologies) supplemented with 1% BSA, and incubated with the indicated antibodies at 4°C overnight. The sections were incubated with Cy2 or Cy3-conjugated secondary antibodies.

Hematoxylin and eosin (H&E) and Sirius Red staining

Liver paraffin sections were stained with H&E or 0.1% Sirius Red (Sigma, 365548) and 0.1% Fast Green (Sigma, F7252) (dissolved in saturated picric acid).

TUNEL assays

Liver frozen sections were fixed with 4% paraformaldehyde and subjected to TUNEL assays using an *In Situ* Cell Death Detection Kit (Roche Diagnostics, Indianapolis, IN, 11684817910), following manufacturer's recommended procedures.

Flow cytometry

The liver was perfused with phosphate buffered saline (PBS) via the portal vein to wash out RBCs, dissected, and mechanically disrupted between two frosted microscope sides. Liver homogenates were filtered through a 100 μm cell strainer, resuspended in 40% isotonic Percoll solution, loaded on the top of a 70% Percoll solution, and centrifuged at 970 × g at room temperatures with no brakes for 30 min. Non-parenchymal cells were collected at the interface of the two Percoll layers, washed, and resuspended in PBS supplemented with 2% FBS. Splenic RBCs were lysed by incubation of cells in 1.66% NH_4Cl solution at room temperature for 10 min. Cells (~2 × 10^6 in 100 μl) were stained with the indicated antibodies in the presence of anti-FcγR antibody 2.4 G2 (blocking nonspecific binding sites). Cells were also stained with either propidium iodide or live-dead fixable dye to exclude dead cells from live cells. Surface-stained cells were fixed, permeabilized, and then stained with antibodies against cytokines using Cytofix/Cytoperm kit (BD Bioscience) or antibody against FoxP3 using the Foxp3/Transcription Factor Staining Buffer Kit (eBioscience). Cell fluorescence was assessed using FACSCanto II (BD Biosciences), and data were analyzed with FlowJo software (version 9.7; Tree Star, Ashland, OR). For analysis, forward and side scatter parameters were used for exclusion of doublets. Antibody information was listed in the CTAT table.

Statistical analysis

Data were presented as means ± SEM. Differences between groups were analyzed with two-tailed Student's *t* test. $p < 0.05$ was considered statistically significant.

For further details regarding the materials used, please refer to the CTAT table.

Results

Deletion of NIK results in growth retardation, hypoglycemia, and premature death

To conditionally delete *NIK*, two loxP sites were inserted into the *NIK* loci (Fig. 1A). Cre-mediated deletion is expected to excise five